MOLECULAR AND CELLULAR BIOLOGY, July 1986, p. 2436–2442
0270-7306/86/072436-07$02.00/0

Vol. 6, No. 7

Mutational Analysis of the Coordinate Expression of the Yeast tRNA^{Arg}-tRNA^{Asp} Gene Tandem

VICENTE M. REYES, ANDREW NEWMAN,† AND JOHN ABELSON*

Divisions of Biology and Chemistry, California Institute of Technology, Pasadena, California 91125

Received 9 December 1985/Accepted 31 March 1986

tRNA genes occur in the yeast genome as highly dispersed and independent transcriptional units. The 5′-tRNA^{Arg}-tRNA^{Asp}-3′ gene tandem, separated by a 10-base-pair spacer sequence, thus represents a rare case of tight clustering. Previous in vitro studies did not reveal any primary transcript from the tRNA^{Asp} gene, but rather a dimeric precursor containing both gene sequences plus spacer, which undergoes a series of maturation steps. This seems anomalous since the tRNA^{Asp} gene contains the sequences necessary for its own transcription. We found that site-directed mutation of the highly conserved C at position 56 to a G in the tRNA^{Arg} gene suppresses all transcription and does not activate the tRNA^{Asp} gene. Precise deletion of the entire tRNA^{Arg} gene gives a similar result. Rescue of tRNA^{Asp} gene transcription is effected either by the precise deletion of both the tRNA^{Arg} gene and spacer or by the precise deletion of this gene with concomitant introduction of an artificial RNA polymerase III start site in the spacer. This artificial start site is ineffective if the tRNA^{Arg} gene is present upstream.

Eucaryotic tRNA genes are transcribed by RNA polymerase III (Pol III) (for a review, see reference 2). The recognition elements that specify transcription of tRNA genes have been shown to reside within the structural gene, although it has been shown that sequences upstream and downstream of the gene can play a role (4, 5, 15, 25). There are two internal control regions (ICRs) of highly conserved primary sequence which define the intragenic promoter of tRNA genes (3, 8, 10, 24): the 5′ ICR, or A-block, which maps to the dihydrouridine loop, and the 3′ ICR, or B-block, which maps to the TψCG loop. It would seem to follow that all of the tRNA genes should be transcribed as monocistronic transcripts since all tRNA genes contain these conserved sequences. This is generally the case. In *Saccharomyces cerevisiae*, for example, there are about 350 tRNA genes, and very few of these are clustered (9). There are, however, several sets of tRNA^{Arg}-tRNA^{Asp} pairs in *S. cerevisiae* (22), and in *Schizosaccharomyces pombe* there is a tRNA^{Ser}-tRNA^{Met} pair (17). In both cases, the two tRNAs are transcribed together to give a dimeric tRNA precursor. Thus, only one of the tRNA genes is normally recognized by the transcription apparatus, and this gene serves as the promoter for the other. To determine which of the genes serves as promoter, we carried out a transcriptional study of the tRNA^{Arg}-tRNA^{Asp} pair using an in vitro system derived from a nuclear extract of *S. cerevisiae* (6, C. S. Parker and J. Topol, unpublished data). We found that linear DNA fragments resulting from restriction endonuclease digestion of the plasmid DNA carrying the tRNA^{Arg}-tRNA^{Asp} genes are excellent templates for RNA synthesis. When restriction sites in the downstream tRNA^{Asp} gene are cleaved, the intact tRNA^{Arg} gene is transcribed, but when the tRNA^{Arg} gene is cleaved, there is no transcription of the intact tRNA^{Asp} gene (12). This experiment showed that the tRNA^{Arg} gene serves as the promoter for the production of the dimeric transcript and further that even when the tRNA^{Arg} gene is inactivated by cleavage, the tRNA^{Asp} gene is incapable of promoting its own transcription.

The question remains: why does the tRNA^{Asp} gene fail to direct its own transcription? We considered three possibilities. (i) The tRNA^{Asp} ICR sequences are intrinsically weak promoter sequences. This hypothesis seems invalidated by the fact that not all tRNA^{Asp} genes are found as tRNA^{Arg}-tRNA^{Asp} pairs. In fact, a monomeric tRNA^{Asp} gene has been isolated which has a sequence identical to that found in the tRNA^{Arg}-tRNA^{Asp} gene tandem (7). Nonetheless, this hypothesis seemed worth testing since it had not been proved that the isolated tRNA^{Asp} gene is transcriptionally active in vivo.

(ii) The binding of a transcription factor to the upstream tRNA^{Arg} gene physically prevents recognition and binding of the transcriptional apparatus to the downstream tRNA^{Asp} gene. It is known that a transcription factor binds specifically and tightly to sequences in the B-block (13). This factor interacts strongly with about 20 base pairs (bp) in the B-block and less tightly to sequences upstream of that. It seems possible that binding of this factor to the upstream tRNA^{Arg} gene could sterically prevent initiation of transcription of the tRNA^{Asp} gene 10 to 15 bp downstream from the binding domain.

(iii) The sequences in the 10-bp intergenic spacer or in the tRNA^{Arg} gene or both may be inhibitory. It is known that certain 5′-flanking sequences to tRNA genes can inhibit transcription (4, 5), although the mechanism of inhibition is not clear.

We used the technique of oligonucleotide-directed mutagenesis (29) to construct precise alterations of the tRNA^{Arg}-tRNA^{Asp} gene pair. These mutants have been used as templates to direct the synthesis of tRNA in a homologous Pol III system (6; Parker and Topol, unpublished data) to determine what alterations allow for independent expression of the tRNA^{Asp} gene.

MATERIALS AND METHODS

pJB19f DNA (1) containing the tRNA^{Arg}-tRNA^{Asp} gene pair and pYSUP6 DNA (11) used as a control template for in vitro transcription experiments have been previously described. All radioisotopes were obtained from Amersham

* Corresponding author.
† Present address: MRC Laboratory of Molecular Biology, Cambridge, CB2 2QH, England.

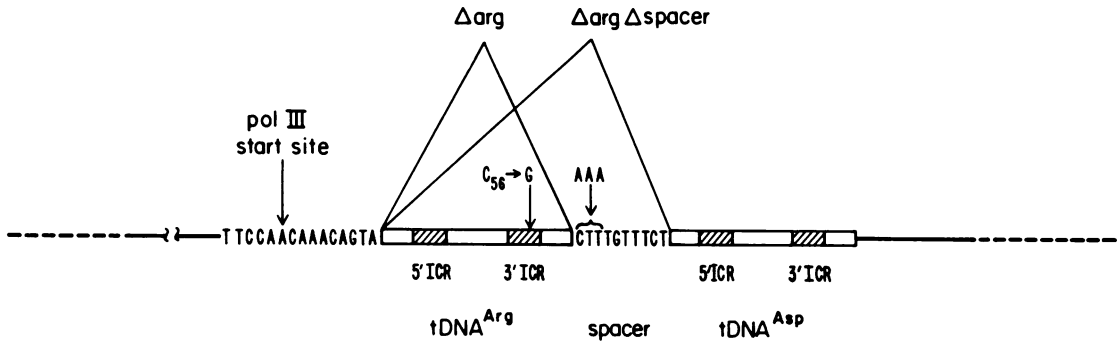

FIG. 1. Organization of the tRNA^{Arg}-tRNA^{Asp} gene system. The tRNA^{Arg} and tRNA^{Asp} genes are shown as hollow boxes, with their A- and B-blocks as hatched regions. Neither gene contains introns. The various mutations are indicated. Each gene is 72 bp long, and the spacer is 10 bp. Yeast flanking regions, shown as thick black lines, are 119 bp upstream of the gene tandem and 45 bp downstream of it. Altogether, the yeast insert is 318 bp cloned into the unique *Sma*I site of the M13mp8 phage vector, which is indicated as broken lines.

Corp. (Arlington Heights, Ill.). Restriction endonucleases were from Boehringer Mannheim Biochemicals (Indianapolis, Ind.), while DNA polymerase I, large fragment (Klenow fragment), T4 DNA ligase, and T4 polynucleotide kinase were from either Bethesda Research Laboratories, Inc. (Gaithersburg, Md.) or New England BioLabs, Inc. (Beverly, Mass.). Mini- and large-scale preparations of double-stranded, replicative-form (RF) DNA were done by the alkaline lysis procedure of Maniatis et al. (16). Large-scale preparation of single-stranded bacteriophage DNA was done by the method of Yamamoto et al. (28). For DNA sequencing, single-stranded phage DNA was recovered from a clarified culture supernatant by polyethylene gycol-NaCl precipitation, followed by phenol extraction. Bacterial transformation was done by the method of Maniatis et al. (16). Polyacrylamide gel electrophoresis for DNA sequencing and transcription reactions were done in 8 or 14% thin polyacrylamide gels containing 7.8 M urea. Dideoxy and chemical sequencing of DNA were done by methods described previously (18, 21). Chemical DNA syntheses either were done manually via phosphotriester chemistry (19) or were made on an Applied Biosystems 380A DNA synthesizer (deoxyoligonucleotides were kindly provided by S. H. Horvath). Yeast nuclear extract used for in vitro transcription experiments was prepared by the method of C. Parker and J. Topol (unpublished data), as described by Engelke et al. (6). Oligonucleotide-directed mutagenesis and in vitro Pol III transcription were performed as previously described (20). DNase I protection analyses (footprinting) were done by the method of Schmitz and Galas (23) as previously described (20), except that radioactive labeling of the DNA fragment was done using [α-^{32}P]dCTP and Klenow fragments instead of [γ-^{32}P]ATP and T4 polynucleotide kinase.

RESULTS AND DISCUSSION

The organization of the tRNA^{Arg}-tRNA^{Asp} gene system is shown in Fig. 1. This gene system had been cloned as a *Hin*dIII yeast fragment of about 3.8 kilobase pairs (2.5×10^6 daltons) in the plasmid pBR313 to give the recombinant plasmid pJB19f (1). A 318-bp fragment containing the tandem gene pair was produced from pJB19f by cleaving it with *Hae*III and *Hpa*I restriction endonucleases. This fragment was cloned into the unique *Sma*I site in the phage vector M13mp8 to obtain a source of single-stranded DNA for oligonucleotide-directed mutagenesis. The orientation of the insert in the phage vector is such that the sequence of the single-stranded DNA is homologous with the sequences of tRNA^{Arg} and tRNA^{Asp}. We refer to this recombinant phage as M13ArgAsp. The double-stranded RF DNA isolated from M13ArgAsp-infected cells served as the template for Pol III transcription of the tRNA^{Arg}-tRNA^{Asp} tandem gene pair. The products of transcription are shown in Fig. 2 (lane 1). The products and extent of transcription from this template were identical to those obtained with pJB19f plasmid DNA (lane 15), so the 318-bp fragment cloned into M13mp8 contains all of the sequences required for synthesis and processing of the two tRNAs.

We have previously characterized the transcription and processing products of the tRNA^{Arg}-tRNA^{Asp} gene system in some detail (6). The largest product, band A, is the de novo transcript consisting of a 5′ leader, tRNA^{Arg}, the 10-bp intergenic spacer, tRNA^{Asp}, and a variable number of 3′ trailer residues including the terminal uridines which specify termination of transcription by Pol III. The de novo transcript is processed in this extract to give the mature tRNA^{Arg} (band D) and tRNA^{Asp} (band C). Band B is the principal intermediate in processing and consists of tRNA^{Arg} flanked by the 5′ leader and spacer sequences. In this extract, the spacer sequence at the 3′ end of the principal intermediate is removed by an exonuclease.

Mutation in tRNA^{Arg} 3′ ICR. In the introduction, we suggested that binding of a transcription factor to the tRNA^{Arg} ICRs could prevent initiation of transcription at the tRNA^{Asp} gene. We have shown with a yeast tRNA$_3^{Leu}$ gene that mutation of the conserved C-56 to G in the TψCG sequence of the 3′ ICR results in a 20-fold decrease in the template activity of the tRNA$_3^{Leu}$ gene in a yeast nuclear extract (20). Previously, it had been shown that an equivalent change in the tRNA^{Tyr} *SUP4* gene results in loss of suppression in vivo and of template activity in vitro (14). The decrease in template activity is correlated with a complete loss of transcription factor binding to the 3′ ICR of the tRNA$_3^{Leu}$ gene as judged by the footprinting assay. We therefore decided to construct a C-56 to G mutation in the tRNA^{Arg} 3′ ICR to test the possibility that this change would unmask the tRNA^{Asp} promoter (mutant designated M13C56G). The mutation was constructed by oligonucleotide-directed mutagenesis (29). The C-56 to G change destroys a *Taq*I restriction site, and this could readily be seen in restriction digests of the RF DNA (data not shown). In addition to this evidence, the C-56 to G change was confirmed by dideoxy sequencing with a synthetic oligonucleotide primer specific for the 3′-flanking region of the tRNA^{Asp}

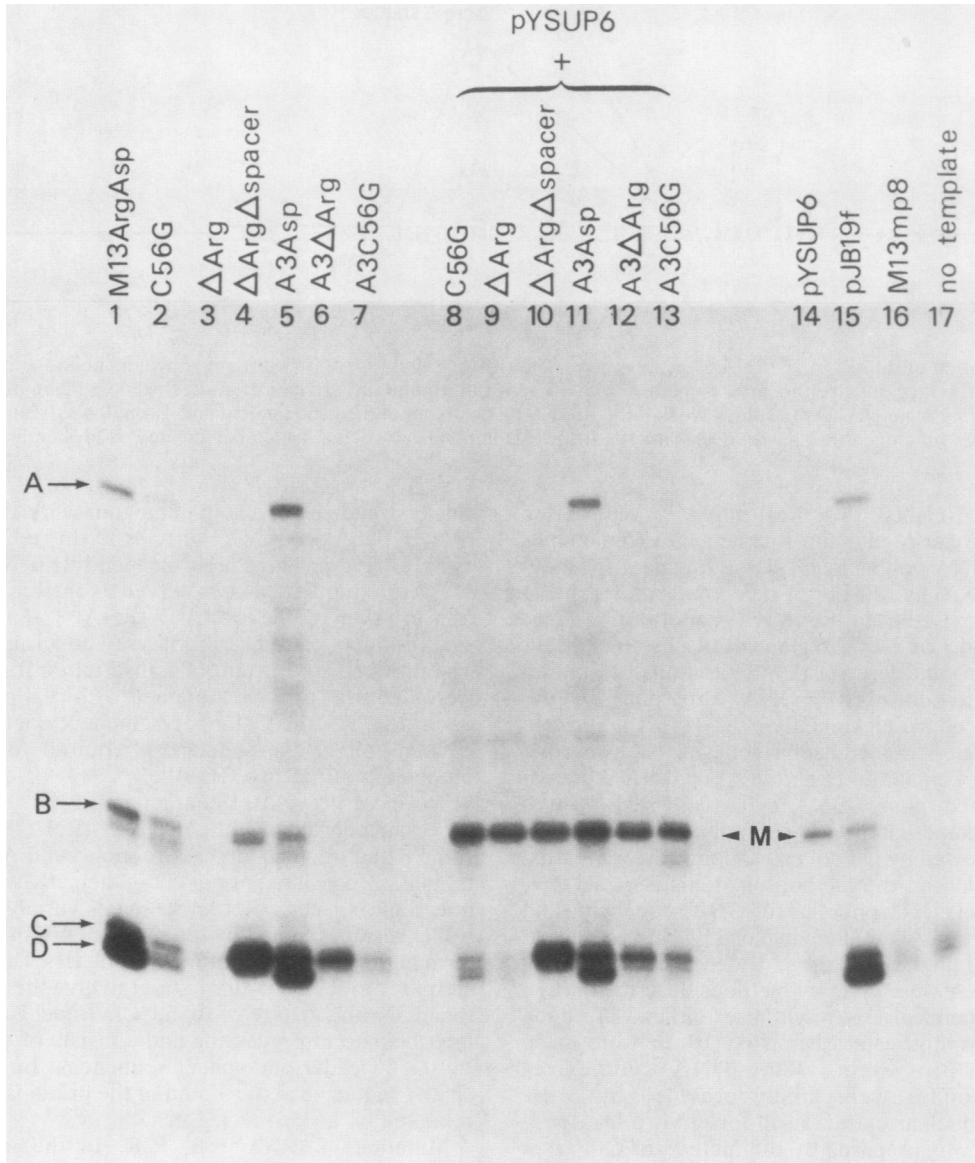

FIG. 2. In vitro transcriptional analysis of the wild-type $tRNA^{Arg}$-$tRNA^{Asp}$ gene system and its various mutant variants. Detailed protocol for the transcription assay is in Materials and methods. The DNA templates are indicated at the top of each lane. Lanes 8 to 13 are mixed-template transcription controls, such that to each corresponding template in lanes 1 to 7 has been added an equal amount of pYSUP6 DNA. The *SUP6* transcript has a size distinct from that of either $tRNA^{Arg}$ or $tRNA^{Asp}$. Band A is the primary dimeric transcript containing the two tRNAs joined together by the spacer sequence, including a 5′ leader and a 3′ trailer sequence. Band B is a major processing intermediate which contains $tRNA^{Arg}$ flanked by the 5′ leader sequence and the spacer sequence. Bands C and D are the mature $tRNA^{Asp}$ and $tRNA^{Arg}$ molecules, respectively. Band M is the pYSUP6 transcript.

gene. The sequences of all mutations described below were confirmed by the same procedure.

When M13C56G RF DNA was used as the template in the nuclear extract, it was found that the C-56 to G mutation results in decrease in template efficiency of about 20-fold when compared with that of M13ArgAsp (Fig. 2, lane 2). This is similar to the effect of the same mutation in the $tRNA_3^{Leu}$ gene. To exclude the possibility that there is an inhibitory substance in the M13C56G reaction mixture, DNA mixing experiments were performed in which an equal amount of pYSUP6 DNA containing the $tRNA^{Tyr}$ *SUP6* gene (which contains a 14-bp intron) was added to the reaction mixture. Transcription of this DNA produced unspliced products which were distinct in size from the mature $tRNA^{Arg}$-$tRNA^{Asp}$ transcripts (Fig. 2, lane 14). This control was performed with each of the mutants, and in no case was the transcription of the pYSUP6 gene inhibited (Fig. 2, lanes 8 to 13).

The C-56 to G change is believed to inactivate the $tRNA^{Arg}$ gene transcription by preventing transcription factor binding to its 3′ ICR. This has been demonstrated directly in the $tRNA^{Arg}$-$tRNA^{Asp}$ gene system by doing DNase I protection assays (23) on the wild-type M13ArgAsp DNA and the C-56 to G mutant (Fig. 3). In the wild-type M13ArgAsp DNA, a footprint (absence of bands) was readily visible in and around the B-block of the $tRNA^{Arg}$

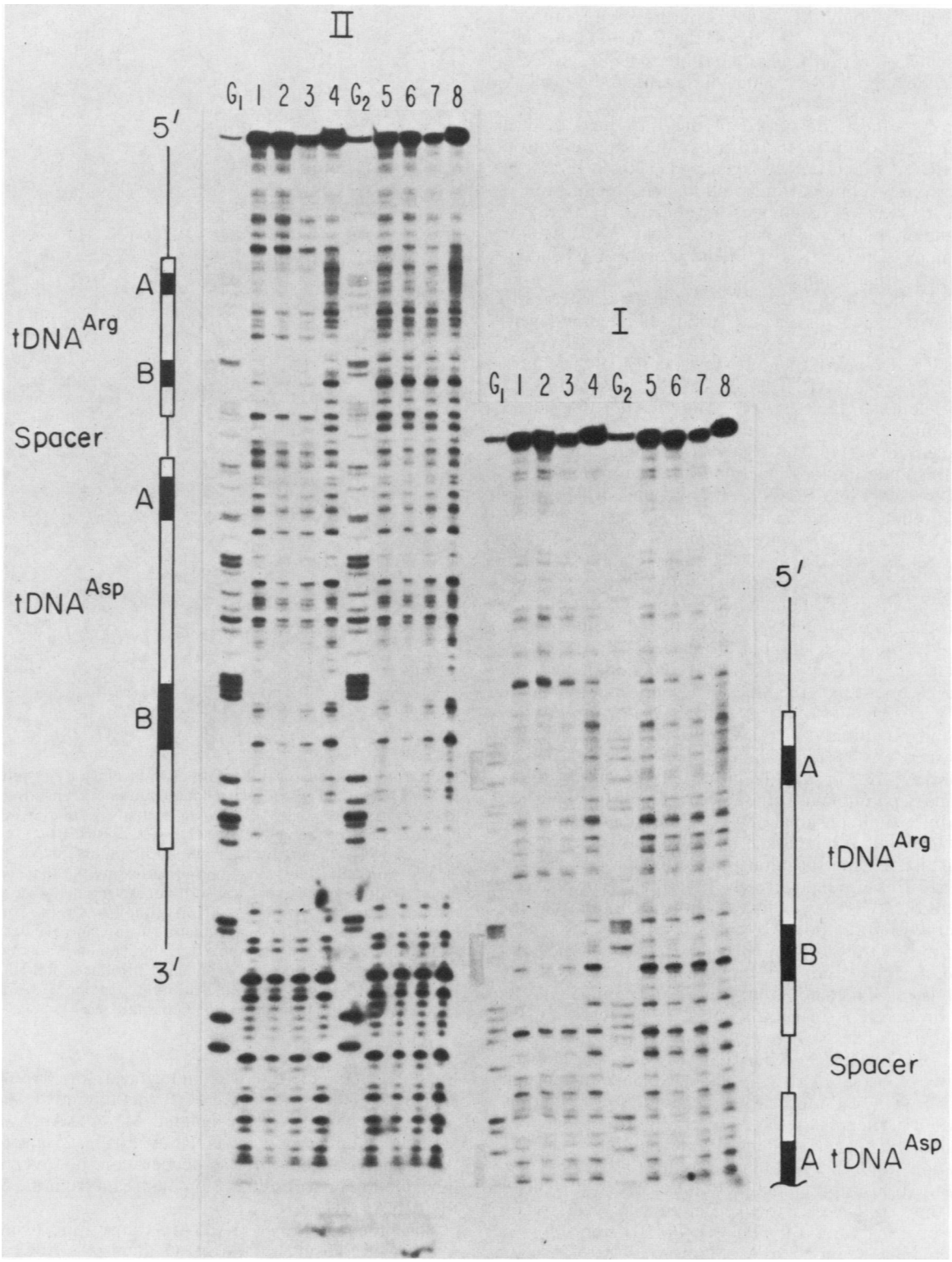

FIG. 3. DNase I protection analysis of M13C56G. A 333-bp *Eco*RI-*Acc*I restriction fragment labeled at the 3′ end (*Acc*I end) and carrying the $tRNA^{Arg}$-$tRNA^{Asp}$ gene tandem was subjected to the footprint assay of Schmitz and Galas (23) (see Materials and methods). Two gel loadings were done to visualize more clearly the transcription factor-binding activity of the $tRNA^{Arg}$ gene. The lanes headed by I constitute the first loading; those headed by II constitute the second loading. Lanes G_1 and G_2 are the Maxam-Gilbert G lanes of M13ArgAsp and M13C56G, respectively. Lanes 1, 2, and 3 are the plus-factor lanes for M13ArgAsp, and lanes 5, 6, and 7 are the corresponding ones for M13C56G. In lanes 1 and 5, the end-labeled fragment was preincubated with 1 μl of transcription factor solution per reaction before DNase I treatment; in lanes 2 and 6, 2 μl; and in lanes 3 and 7, 3 μl. Lanes 4 and 8 are the minus-factor control lanes for M13ArgAsp and M13C56G, respectively, in which the transcription factor preincubation step was omitted. The positions of the $tRNA^{Arg}$ and $tRNA^{Asp}$ genes, together with their A- and B-blocks, are indicated by the adjoining diagrams.

gene, as well as around its A-block, but no such footprints were visible in the $tRNA^{Arg}$ gene of the C-56 to G mutant. There is some protection around the B-block sequence of the $tRNA^{Asp}$ gene of both DNAs, and a little around the A-block sequence. These results immediately eliminate the steric hindrance hypothesis described in the Introduction. The C-56 to G mutation in the B-block has this same effect on both the $tRNA_3^{Leu}$ and $tRNA^{Tyr}$ genes (14, 20). It therefore appears that prevention of transcription factor binding to the 3′ ICR of the $tRNA^{Arg}$ gene does not unmask the $tRNA^{Asp}$ gene promoter, but it remains possible that the 5′ ICR or other sequences in the $tRNA^{Arg}$ gene prevent independent $tRNA^{Asp}$ gene transcription.

Precise deletion of the $tRNA^{Arg}$ gene and the spacer sequence. The technique of oligonucleotide-directed mutagenesis can be used to produce precise deletions as well as point mutations (27). To provide a more stringent test of the effects of the $tRNA^{Arg}$ gene on $tRNA^{Asp}$ expression, we constructed a precise deletion of the $tRNA^{Arg}$ gene. This deletion joined the 5′-flanking sequences of the $tRNA^{Arg}$ gene directly to the 10-bp spacer region (Fig. 1). RF DNA (designated M13ΔArg) carrying this mutation was inactive in transcription (Fig. 2, lane 3). No detectable $tRNA^{Asp}$ was produced. The transcription products were identical to those seen when the control M13mp8 RF DNA was used as the template (Fig. 2, lane 16). Since a wild-type 5′-flanking region and both $tRNA^{Asp}$ gene ICRs were present in this inactive arrangement, we considered that sequences in the spacer could be having an inhibitory effect. To test this hypothesis, we constructed a precise deletion of the spacer region, together with the entire $tRNA^{Arg}$ gene. This deletion joined the 5′-flanking sequence of the $tRNA^{Arg}$ gene directly to the $tRNA^{Asp}$ gene. The RF DNA (designated M13ΔArgΔspacer) carrying this mutation was transcriptionally active, and the product was $tRNA^{Asp}$ (Fig. 2, lane 4). This was confirmed by elution of the tRNA product from the gel, digestion with T_1 RNase, and separation and characterization of the digestion products by two-dimensional thin-layer chromatography (26) (data not shown). A precursor band was also seen in the M13ΔArgΔspacer transcription, and although we did not characterize this product further, its size, about 10 bases larger than $tRNA^{Asp}$, is consistent with the notion that the start site used for wild-type dimer transcription is also utilized for the transcription of the $tRNA^{Asp}$ monomeric gene. We conclude from this experiment that the $tRNA^{Asp}$ promoter is, as expected, transcriptionally competent. Its activity, however, is masked when it is preceded by the 10-bp spacer sequence. This result emphasizes the importance of 5′-flanking sequences in tRNA gene transcription by Pol III. We considered two possible explanations for the inhibitory effects of the spacer sequence on $tRNA^{Asp}$ transcription. First, there is the ever-present "poison sequence" hypothesis. The spacer sequence could possibly assume a configuration which prevents initiation by Pol III or recognition by the transcription factors. Alternatively, it is possible that the sequence of the spacer is inappropriate for initiation of transcription by Pol III. Pol III transcripts in yeasts generally begin with A. Many yeast tRNA genes contain the sequence $(Py)_3CAACAAA$ in the 5′-flanking region (6). Initiation occurs at an A residue about 10 bases upstream from the mature 5′ end of the tRNA. The spacer sequence does not contain any A residue. We elected to test the possibility that the introduction of a string of A residues into the spacer region in M13ΔArg can activate transcription of the $tRNA^{Asp}$ gene.

Introduction of three A residues into the spacer sequence. By oligonucleotide-directed mutagenesis of M13ΔArg, the spacer sequence CTTTGTTTCT was altered to AAATGT TTCT. The RF DNA (designated M13A3ΔArg) was transcribed, and the product was $tRNA^{Asp}$ (Fig. 2, lane 6). Thus, the negative effect of the spacer can be overcome by introduction of A residues 8 bp upstream from the 5′ end of the $tRNA^{Asp}$ gene.

The precursor for M13A3ΔArg transcription was visible upon overexposure of the gel (5 days at −70°C) and was about 8 to 10 bp larger than the mature product ($tRNA^{Asp}$) as judged by their relative electrophoretic mobilities (Fig. 4). It also comigrated exactly with the precursor tRNA for M13ΔArgΔspacer transcription. Although we did not characterize this product further, these results strongly suggest that the artificially introduced start site is functional in M13A3ΔArg.

The artificial Pol III start site was also introduced into the

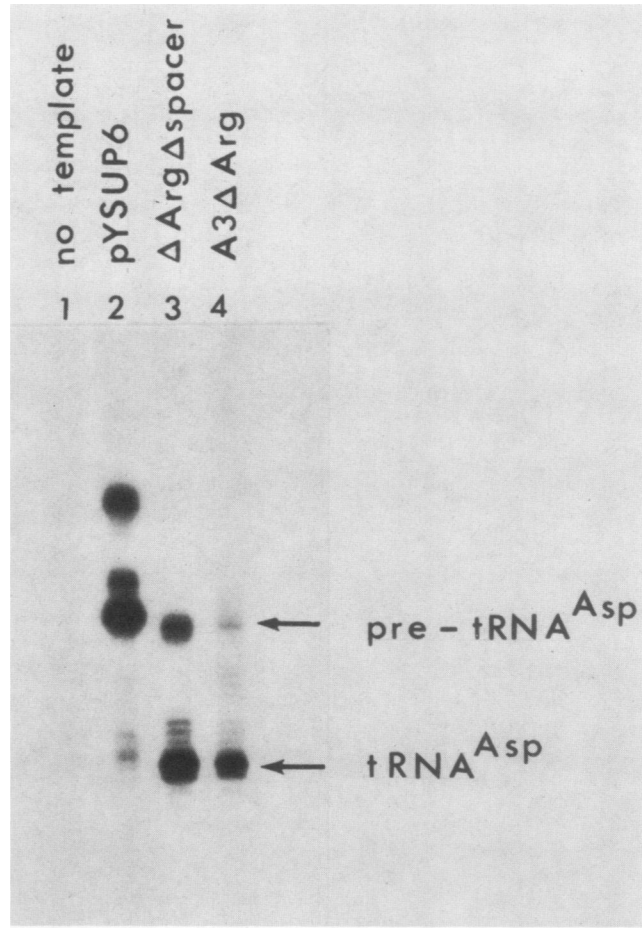

FIG. 4. Evidence of utilization of the artificially introduced Pol III start site in the M13A3ΔArg double mutant. Transcription of the M13ΔArgΔspacer and M13A3ΔArg mutants were run in parallel. Overexposure of the gel (5 days at −70°C) reveals that the precursors for $tRNA^{Asp}$ produced from both mutants have the same electrophoretic mobility and are most probably the same size (lanes 3 and 4). It is known that transcription starts at the A residue at position −10 in the wild-type M13ArgAsp (6). On the basis of the relative sizes of precursor and mature transcript, we assume that this is the same start site utilized for precursor production in the M13ΔArgΔspacer. Since the artificially introduced Pol III start site is at position −10 relative to the $tRNA^{Asp}$ gene in M13A3ΔArg, we conclude that it is indeed the one being utilized.

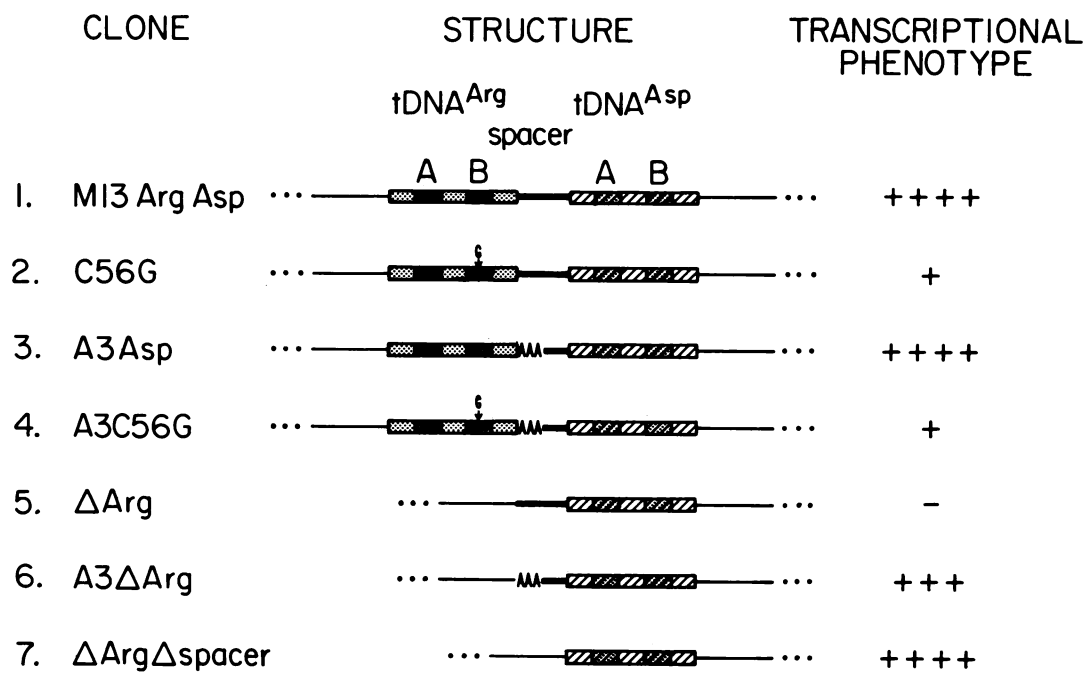

FIG. 5. Summary of results. The structures of the various mutants under study are indicated. The introduction of a G residue in the B-box of the $tRNA^{Arg}$ gene is indicated. The artificially introduced Pol III start site is indicated as AAA. Levels of transcription in vitro are indicated as plus signs, with wild-type level designated as ++++ and no transcription being −. These designations are all in reference to Fig. 2.

intergenic spacer of the wild-type gene tandem (mutant designated A3Asp) and into the C-56 to G mutant (designated A3C56G). Transcription of the wild-type gene was not affected by this alteration of the spacer sequence (Fig. 2, lane 5). Since the spacer sequence inhibited transcription of $tRNA^{Asp}$ in the ΔArg construct, it is possible that it is the spacer which inhibits the transcription of $tRNA^{Asp}$ in the C56G construct. If so, introduction of A3 into the intergenic spacer of C56G should activate $tRNA^{Asp}$ transcription. This, however, was not the case (Fig. 2, lane 7). Apparently, the inhibition of the $tRNA^{Asp}$ gene promoters in the tandem construct is complex.

The results of this study are summarized in Fig. 5. Independent transcription of the $tRNA^{Asp}$ gene was achieved by precise deletion of the $tRNA^{Arg}$ gene together with the 10-bp spacer. Deletion of the $tRNA^{Arg}$ gene sequence by itself was not sufficient to activate transcription of the $tRNA^{Asp}$ gene, suggesting an inhibitory effect of the spacer sequence. This inhibition was partially overcome by the introduction of three A residues at the 5′ edge of the intergenic spacer. The failure, however, of this sequence to activate transcription of $tRNA^{Asp}$ in the $tRNA^{Arg}$ C-56 to G mutant suggests that the $tRNA^{Arg}$ sequences, as well as the spacer region, inhibit the utilization of the $tRNA^{Asp}$ gene promoters in the tandem by Pol III. Therefore, it must be the sequence of the $tRNA^{Arg}$ gene together with the spacer sequence which suppresses an otherwise competent $tRNA^{Asp}$ intragenic promoter.

ACKNOWLEDGMENTS

We thank Peter Johnson, David Engelke, and Marjorie Strobel for their help and for useful discussions.

This work was supported by a grant from the American Cancer Society (NP302D) and Public Health Service grant GM30356 from the National Institutes of Health.

LITERATURE CITED

1. **Beckmann, J. S., P. F. Johnson, and J. N. Abelson.** 1977. Cloning of yeast transfer RNA genes in *Escherichia coli*. Science **196**:205–208.
2. **Ciliberto, G., L. Castagnoli, and R. Cortese.** 1983. Transcription by RNA polymerase III. Curr. Top. Dev. Biol. **18**:59–88.
3. **Ciliberto, G., L. Castagnoli, D. A. Melton, and R. Cortese.** 1982. Promoter of a eukaryotic $tRNA^{Pro}$ gene is composed of three noncontiguous regions. Proc. Natl. Acad. Sci. USA **79**:1195–1199.
4. **DeFranco, D., S. Sharp, and D. Soll.** 1981. Identification of regulatory sequences contained in the 5′ flanking region of *Drosophila* lysine $tRNA_2$ genes. J. Biol. Chem. **256**:12424–12429.
5. **Dingermann, T., D. S. Burke, S. Sharp, J. Schaack, and D. Soll.** 1982. The 5′ flanking sequences of *Drosophila* $tRNA^{Arg}$ genes control their in vitro transcription in a *Drosophila* cell extract. J. Biol. Chem. **257**:14738–14744.
6. **Engelke, D. R., P. Gegenheimer, and J. Abelson.** 1985. Nucleolytic processing of a $tRNA^{Arg}$-$tRNA^{Asp}$ dimeric precursor by a homologous component from *Saccharomyces cerevisiae*. J. Biol. Chem. **20**:1271–1279.
7. **Gafner, J., E. M. De Robertis, and P. Philippsen.** 1983. Delta sequences in the 5′ noncoding region of yeast tRNA genes. EMBO J. **2**:583–591.
8. **Galli, G., H. Hofstetter, and M. L. Birnstiel.** 1981. Two conserved sequence blocks within eucaryotic tRNA genes are major promoter elements. Nature (London) **394**:626–631.
9. **Guthrie, C., and J. Abelson.** 1982. Organization and expression of tRNA genes in *Saccharomyces cerevisiae*, p. 487–528. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), Molecular biology of the yeast *Saccharomyces*: metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. **Hofstetter, H., A. Kressmann, and M. L. Birnstiel.** 1981. A split promoter for a eucaryotic tRNA gene. Cell **24**:573–585.
11. **Johnson, P. F., and J. Abelson.** 1983. The yeast $tRNA^{Tyr}$ gene intron is essential for correct modification of its tRNA product. Nature (London) **302**:681–687.

12. **Kjellin-Straby, K., D. R. Engelke, and J. Abelson.** 1984. Homologous in vitro transcription of linear DNA fragments containing the $tRNA^{Arg}$-$tRNA^{Asp}$ gene pair from *Saccharomyces cerevisiae*. DNA **3**:167–171.
13. **Klemenz, R., D. J. Stillman, and E. P. Geiduschek.** 1982. Specific interactions of *Saccharomyces cerevisiae* proteins with a promoter region of eukaryotic tRNA genes. Proc. Natl. Acad. Sci. USA **79**:6191–6195.
14. **Koski, R. A., D. S. Allison, M. Worthington, and B. D. Hall.** 1982. An in vitro RNA polymerase III system from *S. cerevisiae*: effects of deletions and point mutations upon SUP4 gene transcription. Nucleic Acids Res. **10**:8127–8143.
15. **Larson, D., J. Bradford-Wilcox, L. S. Young, and K. U. Sprague.** 1983. A short 5′ flanking region containing conserved sequences is required for silkworm alanine tRNA gene activity. Proc. Natl. Acad. Sci. USA **80**:3416–3420.
16. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Mao, J., O. Schmidt, and D. Soll.** 1980. Dimeric transfer RNA precursors in *S. pombe*. Cell **21**:509–516.
18. **Maxam, A. M., and W. Gilbert.** 1977. A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA **74**:560–564.
19. **Miyoshi, K., T. Huang, and K. Itakura.** 1980. Solid-phase synthesis of polynucleotides. III. Synthesis of polynucleotides with defined sequences by the block-coupling phosphotriester method. Nucleic Acids Res. **8**:5491–5505.
20. **Newman, A. J., R. C. Ogden, and J. Abelson.** 1983. tRNA gene transcription in yeast: effects of specified base substitutions in the intragenic promoter. Cell **35**:117–125.
21. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
22. **Schmidt, O., J. Mao, R. Ogden, J. Beckmann, H. Sakano, J. Abelson, and D. Soll.** 1980. Dimeric tRNA precursors in yeast. Nature (London) **287**:750–752.
23. **Schmitz, A., and D. J. Galas.** 1983. The study of protein-DNA binding specificity: DNAse footprinting, p. 305–347. *In* S. M. Weissman (ed.), Methods of DNA and RNA sequencing. Praeger Press, New York.
24. **Sharp, S., D. DeFranco, T. Dingermann, P. Farrell, and D. Soll.** 1981. Internal control regions for transcription of eucaryotic tRNA genes. Proc. Natl. Acad. Sci. USA **78**:6657–6661.
25. **Sprague, K. U., D. Larson, and D. Morton.** 1980. 5′ flanking sequence signals are required for activity of silkworm alanine tRNA genes in homologous in vitro transcription systems. Cell **22**:171–178.
26. **Volckaert, G., and W. Fiers.** 1977. Micro thin layer techniques for rapid sequence analysis of ^{32}P-labeled RNA: double digestion and pancreatic ribonuclease analyses. Anal. Biochem. **83**:228–239.
27. **Wallace, R. B., P. F. Johnson, S. Tanaka, M. Schöld, K. Itakura, and J. Abelson.** 1980. Directed deletion of a yeast transfer RNA intervening sequence. Science **209**:1396–1400.
28. **Yamamoto, K. R., B. M. Alberts, R. Benzinger, L. Lawhorne, and G. Treiber.** 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large scale virus purification. Virology **40**:734–744.
29. **Zoller, M. J., and M. Smith.** 1982. Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any fragment of DNA. Nucleic Acids Res. **10**:6487–6500.